Original Papers Plant Tissue Culture Letters, **12**(3), 267-272(1995)

Enhancement of Seed Germination and Protocorm Formation in *Calanthe discolor* (Orchidaceae) by NaOCl and Polyphenol Absorbent Treatments

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(Received August 16, 1994)
(Accepted August 12, 1995)

The effects of sodium hypochlorite and polyphenolic absorbent on seed germination and protocorm formation of *Calanthe discolor* Lindl. were investigated. Mature seeds of *Calanthe discolor* Lindl. were aseptically collected from undehisced pods and sowed with or without the treatment of 1% sodium hypochlorite solution. About 30% of the seeds treated with NaOCl were at germination stage (rupture of the seed coat) or protocorm stage whereas only 9% of the seeds were at the same stage without the treatment. Embryos in 79% of the seeds treated with NaOCl solution were stained by soaking in 1% T. T. C. (2, 3, 5-triphenyl tetrazolium chloride) solution for one day following the four days of culture in the nutrient medium, whereas only 24% of the embryos were stained without the treatment. The addition of 10% Polyclar-AT®, a polyphenolic absorbent, in the medium enhanced protocorm formation. About 25% of the seeds formed protocorms in the medium containing Polyclar-AT®. The addition of Polyclar-AT® also prevented browning of protocorms which occurred after prolonged incubation in culture medium.

Introduction

Terrestrial *Calanthe* species are popular in Japan as ornamental orchids and many cultivars have been produced by intra- and interspecific hybridization. However, the breeding and seed propagation of *Calanthe* species are always hindered by the difficulty in seed germination.

Although the mechanism involved in the difficulty in seed germination of *Calanthe* species is still fragmental and obscure, it has been suggested that the dormancy is induced by the accumulation of inhibitory substances[1] or by increasing impermeability of the embryo during seed maturation[2].

For breaking seed dormancy, sodium hypochlorite has been reported to be effective for some plant species[3-7]. Hypochlorite, such as NaOCl and $Ca(ClO)_2$, solution has been commonly used for the sterilization of orchid seeds, and it has also been reported to have stimulatory effect on seed germination in some orchid species[8-10].

Beside dormancy, cessation and/or death of the embryos and protocorms during the early stages of germination are known to be other important problems in seed propagation of *Calanthe* species. The necrosis or browning is a widely known phenomenon in *in vitro* culture of orchids and is considered to be caused by oxidation of polyphenolic substances[11-13]. Therefore, polyphenolic absorbents are expected to prevent such browning or necrosis in germinating orchid seeds and seedlings.

In the present study, attempts were made to induce seed germination of *Calanthe discolor* asymbiotically by the pre-treatment of brief immersion in NaOCl solution, and to stimulate protocorm formation and to prevent the browning during the culture by adding polyphenol absorbents into the culture medium. The possible mechanism of difficulty in seed germination and protocorm formation of this species is also discussed.

Materials and Methods

Mature seeds of *Calanthe discolor* were routinely collected from undehisced pods during late October to late November. The seeds were kept at 5°C in an airtightened sample tube without desiccant until use. The seeds were sterilized with 1% NaOCl solution in a small sample tube which was agitated by hand for 7 min. followed by rinsing 5 times with sterilized distilled water. Detailed method of sterilization was described in our previous paper[2].

The seeds were also sown without sterilization with NaOCl. In this case, seeds were aseptically taken out from the pods using blade and forceps after surface-sterilization of the pods with 70% ethyl alcohol for 30 sec. and with 1% NaOCl for 10 min. successively, followed by five rinses with sterilized distilled water. These aseptically collected seeds were also stored in sterilized sample tubes until use. When these seeds were sown, they were directly put into agar-solidified culture medium of Ichihashi and Yamashita[14] form which some minor elements were excluded, or washed with sterilized distilled water 5 times before the culture.

In the experiment on polyphenol absorbents, seeds were sown in glass tubes containing the medium of Ichihashi and Yamashita[14] from which agar and some minor elements were excluded[2]. For the experiment to test the effects of polyphenol absorbents on seed germination and protocorm growth, 10% (w/v) P. V. P. (polyvinylpyrrolidone) or Polyclar-AT® (dimer of polyvinylpyrrolidone; ISP Technology INC. N. J., U. S. A.) was added to medium. The former is soluble and the latter

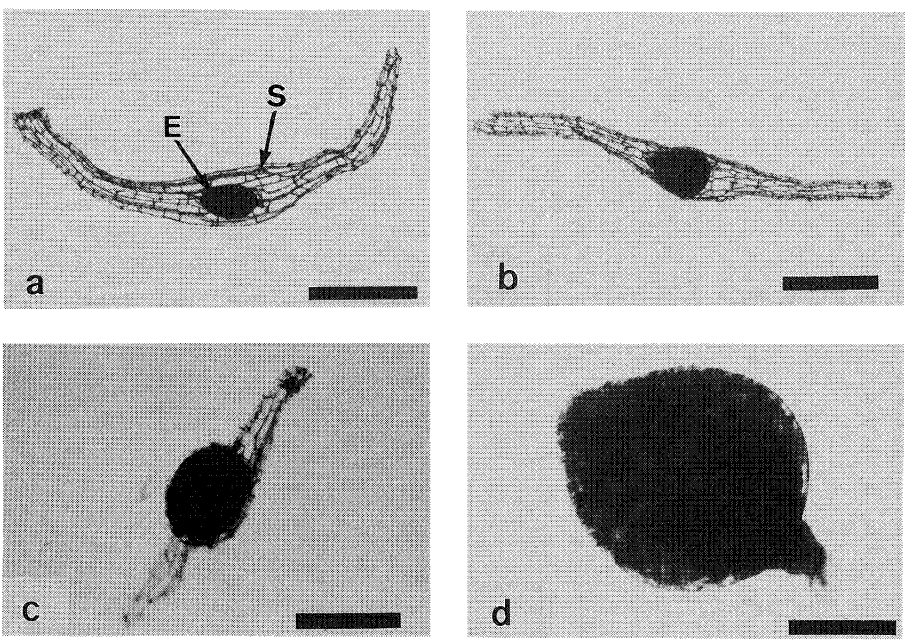

Fig. 1 The successive developmental stages of *Calanthe discolor* seed germination and protocorm formation.
a, No germination stage (bar=30 μm). b, Pre-germinaiton stage (bar=30 μm). c, Germination stage (bar=50 μm). d. Protocorm stage (bar=8 mm). S: Seed coat. E: Embryo.

insoluble in the medium. Each tube containing 12 m*l* of nutrient solution was sealed with aluminum foil. The seeds were cultured under the same conditions as those described in our previous paper[2]. About 400-600 seeds were put in a tubes and at least six replications were made for each treatment.

The activity of the seeds was determined by the percentage of red-stained embryos after soaking at least 400 seeds in 1% T. T. C. (2, 3, 5-triphenyl tetrazolium chloride) solution for 24 hr at 35°C in the dark. The process of seed germination was divided into following four categories according to the developmental stage of embryos.

1. "No germination" stage (**Fig. 1-a**). No growth of embryo occurs.
2. "Pre-germination" stage (**Fig. 1-b**). Embryo swells in the width of seed coat.
3. "Germination" stage (**Fig. 1-c**). Embryo emerges from the seed coat.
4. "Protocorm" stage (**Fig. 1-d**). Embryo is completely discharged from the seed coat.

Determination of these stages was conducted under a stereoscopic microscope after taking at least 1, 000 seeds out from three tubes.

Results

Effect of sodium hypochlorite on the seed activity and germination

Table 1 shows the rate of *Calanthe* embryos stained by T. T. C. after immersing the seeds in 1% NaOCl solution for 7 min. About 50% of the embryos showed red coloration after the treatment, but only 3% were stained in non-treated ones. With 4 days of culture in the nutrient medium, the rate of the seeds with stained embryos increased in both treated (79%) and non-treated seeds (24%).

About 30% of the seeds treated with NaOCl but about 9% of those without treatment developed further than "germination" stage after 280 days of culture on agar-solidified medium (**Fig. 2**). The seeds only washed with 5 changes of distilled wather responded like non-treated ones.

Stimulation of protocorm formation by polyphenol absorbent

As shown in **Fig. 3**, the addition of Polyclar-AT® to the culture medium was effective in protocorm formation. Though the percentages of the seeds which started to germinate were almost equal between the culture with Polyclar-AT® (88%) in the medium and that without it (91%), rate of protocorm formation in the former was 5 times higher than the latter. However a soluble polyphenol absorbent, P. V. P. (polyvinylpyrrolidone), had an inhibitory effect on the induction of seed germination. About forty percent of the seeds were at the no germination stage and no protocorm was observed in the P. V. P. medium. In the control culture, the most of fully-grown green protocorms eventually turned brown at 7 months of culture. In contrast most of the

Table 1. The effects of 1% NaOCl treatment for 7 min. on the stainability of *Calanthe discolor* seeds.

	Days in culture	
NaOCl Treatment	0 day	4 day
None	3	24 (%)
1%, 7 min.	53	79

The seeds treated with or without NaOCl solution were soaked in 1% T. T. C. solution for 1 day following the culture in the liquid medium for 0 or 4 days.

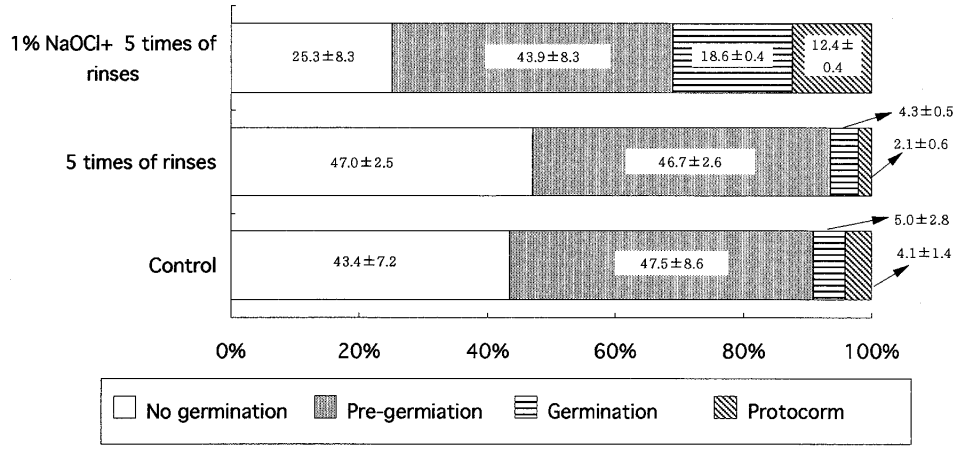

Fig. 2 The effects of 1% NaOCl solution for 7 min. on seed germination of *Calanthe discolor*. The seeds were cultured for 280 days on the agar-solidified medium of Ichihashi and Yamashita from which some minor elements were exculuded. Each value represents the percentage±S. D.

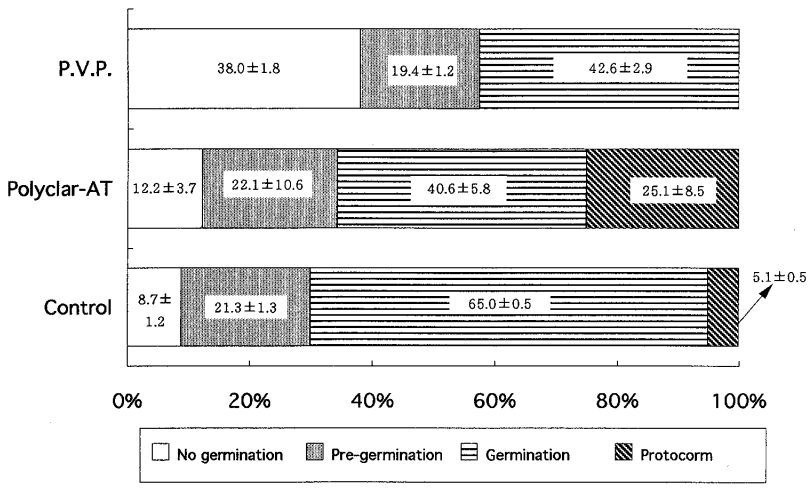

Fig. 3 The effects of polyphenol absorbents Polyclar-AT® and P. V. P. (polyvinylpyrrolidone) on seed germination of *Calanthe discolor*.
The seeds were treated with 1% NaOCl solution for 7 min. (washed 5 times with sterilized distilled water) and cultured in liquid medium[2] for 220 days. Each value represents the percentage±S. D.

protocorms grown Polyclar-AT® remained dark green after the same period of culture. Seeds which are treated with 1% NaOCl and cultured in liquid medium resulted in more rapid germination compared with those on agar. More than 70% of the seed developed into germination and protocorm stages in 220 days in liquid medium, whereas 31% in 280 days on agar solidified medium (**Figs. 2** and **3**).

Discussion

Sterilization of seeds is a prerequisite for asymbiotic culture of orchid seed, unless the seeds were harvested and stored aseptically. Therefore, the effect of a commonly used sterilizing agent, NaOCl on germination of *Calanthe* seeds was examined using the seeds aseptically collected.

Sodium hypochlorite seems to have the effect of breaking seed dormancy of *Calanthe* because T.

T. C. stainability as well as germination rate were increased by the treatment (**Fig. 1** and **Table 1**). The effectiveness of hypochlorite solution for breaking seed dormancy has also been reported in other species such as *Stipa*[3], *Bouteloua*[6], *Avena*[7] and some Western European orchids[10]. In these studies, the mechanism of action of NaOCl for inducing seed germination or breaking dormancy has been considered as the partial degradation of the seed coat and/or the solubilization and oxidation of inhibitor(s)[3,4,7]. In *Calanthe,* however, no visual difference of seed coat was observed between the treated and non-treated seeds. The presence of inhibitor(s) is also not evident in the seed of this species. Therefore, the mechanism of action of NaOCl for breaking seed dormancy in *Calanthe* remains as a subject for further study.

Our preliminary observation suggests that permeability of the seed may decrease during maturation, because the seeds harvested at early stages of maturation in mid-September sunk in NaOCl solution within a minute, whereas those fully matured collected in early-December remained a float in the solution. In the previous study[2], we suggested that the permeability of the embryo may play an important role in seed germination of this species. It is possible that the sensitivity to NaOCl may change due to the difference in the permeability of the seeds with different degrees of maturation. Further investigations will be needed for clarifying this possibility.

T. T. C. activity has routinely been used as a good indicator of germinability of the seeds[15]. In the present study on *Calanthe* seeds, however, T. T. C. activity can be used as the indicator for the activation of embryos for germination (**Table 1** and **Fig. 2**). This might be useful in clarifying the effect of certain treatments on the enhancement of germination in hard-to-germinate orchid species.

Effectiveness of Polyclar-AT® in the medium on preventing both the browning of protocorms and the inhibition of protocorm formation suggests that these growth depressions may be induced by polyphenolic substance(s) which were secreted by the seed and protocorms during the culture. The growth of germinating seeds and/or protocorms may be hindered by the substance(s) produced by themselves during the prolonged culture. Therefore, continuous change of the culture medium will be another way to avoid these growth inhibitions.

In tobacco anther culture, pollen plantlet formation was enhanced by P. V. P.[16]. In *Calanthe,* however, only Polyclar-AT® was effective for enhancing protocorm formation and P. V. P. acted inhibitorily against expectation. As P. V. P. is soluble in water, the high concentration used in the present experiment increased osmotic pressure of the medium which might act inhibitorily for seed germination.

From the results of the present study, it is assumed that the difficulty in germinating *Calanthe* seeds is due to (1) primary dormancy that persists at harvest-time and (2) growth depression of the germinating seeds. The primary dormancy might be broken with NaOCl treatment and the growth depression can be avoided by addition of polyphenol absorbent in the medium.

Further studies will be needed for testing the adaptability of both NaOCl treatment and addition of polyphenol absorbents in the medium for enhancing the seed germination of other so-called hard to germinate orchid species.

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《和文要約》

ラン科植物エビネ(*Calanthe discolor*)における次亜塩素酸ナトリウムならびにポリフェノール吸着剤処理による種子発芽ならびにプロトコーム形成の促進

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次亜塩素酸ナトリウムおよびポリフェノールの吸着剤が，エビネ(*Calanthe discolor* Lindl.)の種子発芽ならびに，プロトコーム形成に及ぼす影響について調査した．完熟種子を裂果前の果実より無菌的に取り出し，無処理のまま，もしくは1%次亜塩素酸ナトリウム水溶液により処理した後に，播種した．1%次亜塩素酸ナトリウム水溶液処理区においては，約30%の種子が，発芽期(種皮より胚が突出)もしくはプロトコーム期にあったが，無処理区における両期の種子は9%にとどまった．4日間培養した後，1% T. T. C.(2, 3, 5-triphenyl tetrazolium chloride)水溶液に1日間の浸漬を行ったところ，同処理区においては79%の種子の胚が染色されたが，無処理区においては24%であった．

培地に10%の，ポリフェノールの吸着剤であるPolyclar-ATを添加すると，プロトコームの形成が促進され，25%の種子がプロトコームを形成した．Polyclar-ATの添加は，発芽後に起こりやすい，プロトコームの褐変も防いだ．